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Cloned DNA probes distinguish endemic and exotic *Entomophaga grylli* fungal pathotype infections in grasshopper life stages

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Abstract

***Entomophaga grylli* is a fungal pathogen of grasshoppers and at least three pathotypes are recognized world-wide. Pathotypes 1 and 2 are endemic to North America while the Australian pathotype 3 had been released into two field sites in North Dakota between 1989 and 1991. Grasshoppers were collected over the summer at the field sites in 1992 and assessed for pathotype infection by cloned DNA probe analysis. The three most predominant grasshopper species that were infected (*Melanoplus sanguinipes*, *M. bivittatus* and *Camnula pellucida*) were assessed for pathotype infection with respect to their life stages (nymphal instars and adult males and females). Pathotype 1 predominantly infected grasshoppers in the subfamilies Oedipodinae and Gomphocerinae and pathotype 2 predominantly infected grasshoppers in the subfamily Melanoplinae. Early-instar *M. sanguinipes* and *M. bivittatus* had higher pathotype 2 infection frequencies, while late-instar and adult *C. pellucida* had higher pathotype 1 infection frequencies. Cross-infection by the pathotypes did occur in up to 3% of the individuals, on a per species basis, and primarily in later instar and adult grasshoppers. Pathotype 3 infections occurred in later instar and adults of the three grasshopper species. Infection of grasshoppers by *E. grylli* pathotypes is discussed with reference to the fungal life cycles.**

Keywords: cloned DNA probes, *Entomophaga grylli*, grasshoppers, exotic fungi, biological control

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Introduction

Entomophaga grylli is a cosmopolitan species of zygomycetous fungus represented by a complex of pathotypes that infect grasshoppers (Soper *et al*. 1983). Three pathotypes have recently been differentiated by random amplification of polymorphic DNA (RAPD) and by genomic probe analysis (Bidochka *et al*. 1995); however, more than three pathotypes are suspected to be found world-wide. Pathotypes 1 and 2 are found in North America while pathotype 3 is found in Australia (Humber 1989). Pathotypes 1 and 2 exhibit restricted host ranges with respect to grasshopper subfamilies; pathotype 1 predominantly infected grasshoppers in the subfamily Oedipodinae, the banded-winged grasshoppers (e.g. *Camnula pellucida*) while pathotype 2 predominantly infected grasshoppers in the subfamily Melanoplinae, the spur-throated grasshoppers (e.g. *Melanoplus sanguinipes*, *M. differentialis*) (Ramoska *et al*. 1988). Pathotype 3 infected grasshoppers of the subfamily Cyrtacanthacridinae in Australia (i.e. *Praxibulus* sp.; Humber 1989). However, laboratory tests showed that pathotype 3 also exhibited a broader host range and infected North American oedipodine and melanopline grasshoppers (Ramoska *et al*. 1988). As part of a grasshopper biocontrol effort, pathotype 3 was released by the USDA-ARS into McKenzie county, North Dakota between 1989 and 1991 (Ramos 1993).

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Infected grasshoppers climb to elevated places, such as stalks of wheat. In this position, the grasshoppers die in sometimes spectacular epizootics in what is known as the 'summit disease' syndrome. These grasshoppers contain resting spores that are morphologically indistinguishable between pathotypes. A method was required to distinguish the exotic fungal pathotype from the endemic pathotypes in order to track its fate and persistence in the field. Environmental concerns have been raised concerning the fate and impact of the exotic fungal pathogen on the prairie ecosystem (Lockwood 1993). We had developed a method by which the resting spores in the infected grasshoppers can be fractured, the DNA extracted and used as a template against pathotype-specific DNA probes (Bidochka *et al*. 1995). The application of this sensitive molecular technique distinguished the three pathotype infections in grasshoppers collected near the pathotype 3 release sites in North Dakota (Bidochka *et al*. 1996). This allowed us to examine the degree to which pathotype 3 persisted in the field.

During our evaluations of pathotype infections in grasshoppers we observed an infection bias with respect to grasshopper life stages by the three pathotypes. Grasshopper life stage infections by the fungal pathotypes is an important factor in the construction of predictive models of grasshopper population dynamics (Carruthers *et al*. 1988). To our knowledge, there are no published accounts of infection by these pathotypes in different grasshopper life stages. Here we address the question of fungal infections with respect to grasshopper life stages utilizing cloned DNA probes to distinguish between the pathotypes.

Materials and Methods

Entomophaga grylli pathotype probes

The construction of DNA probes, methods for resting spore fracturing and hybridization protocols have been reported previously (Bidochka *et al*. 1995, 1996).

Releases of pathotype 3

Grasshoppers (*M. differentialis*) were reared at the USDA-ARS Rangeland Insect Laboratory, Bozeman, Montana, USA and were injected with 10 μL of ≈ 10^4 pathotype 3 protoplasts. Three days after infection, grasshoppers were released at two separate sites. Releases of infected grasshoppers in McKenzie county, N. Dakota were: 500 each at Field 1 on 24 July 1989 and 5, 7, 9, 11, 13 June 1990, but no releases at Field 1 in 1991 or 1992; 500 each at Wold's on 6, 8, 11 and 13 June 1991, but no releases at Wold's in 1992. The locations of the release sites were Wold's Ranch, (T153N, R97W, Sec33) North Dakota, USA and Field 1 (T154N, R955W, Sec32) near Lake Sakakawea, North Dakota, USA.

Collection of grasshoppers

Live grasshoppers were collected by sweep netting every 7–10 days between 1 June and 30 August at and near the release sites in McKenzie county. They were identified to species, instar and adult sex and then brought into indoor facilities where they were fed romaine lettuce, wheat bran, native grasses, and provided with a water source. The grasshoppers were monitored daily and the dead grasshoppers were removed, the species and instar were recorded and the abdomen internally examined for the presence of resting spores. Grasshoppers that did not die within 21 days were destroyed and discarded. The infected grasshoppers were kept frozen and transferred to the Boyce Thompson Institute for Plant Research, Cornell University, for pathotype identification using the DNA probes.

Results

Table 1 shows the representation of grasshopper species, within their respective subfamilies, collected at the two sites. The major difference is that the order of abundance of grasshopper subfamilies at the Wold's site was Gomphocerinae > Melanoplinae > Oedipodinae, while at the Field 1 site it was Melanoplinae > Oedipodinae > Gomphocerinae.

Of the grasshoppers collected, 6.1% and 6.8% were infected by one of three *E. grylli* pathotypes at Field 1 and Wold's, respectively. Table 2 shows data for *E. grylli* pathotype infection in grasshopper species by DNA probe analysis. Over the two sites, *M. sanguinipes*, *M. bivittatus* and *C. pellucida* had the greatest number of infected grasshoppers. Percentage of infection was overall highest by pathotype 2 in *M. bivittatus* at Wold's (Table 2) and this grasshopper species represented ≈ 10% of the grasshoppers collected at that site. Pathotype 1 infections were greatest in the oedipodine (*C. pellucida*) and gomphocerine grasshoppers while pathotype 2 infections were greatest in the melanopline grasshoppers. Although pathotypes 1 and 2 predominantly infected grasshoppers of the subfamilies Oedipodinae and Melanoplinae, respectively, there was up to 3% cross-infection. Pathotype 3 infections were found in all grasshopper subfamilies and infection percentages ranged as high as 8.33% (*Melanoplus infantilis*).

Figure 1 shows the infection of *M. sanguinipes*, *M. bivittatus* and *C. pellucida* life stages at the Field 1 and Wold's sites as a percentage of the number of that life stage collected (five instars and adult male and female) when they died with *E. grylli* infection. Early-instar *M. sanguinipes* showed greater frequencies of infection with pathotype 2 than later instar and adult grasshoppers at both sites (Fig. 1). Pathotype 3 infections occurred primarily in later instar and adult grasshoppers. Adult female *M. bivittatus*

Table 1 Representation of grasshopper species at Wold's and Field 1 in 1992 collected by sweep-netting

Subfamily	Species	Field 1 %	Field 1 (*n*)	Wold's %	Wold's (*n*)
Melanoplinae	*Aeoloplides turnbulli*	0.13	(4)	0	
	Melanoplus sanguinipes	29.12	(876)	17.86	(579)
	M. bivittatus	4.49	(135)	9.93	(322)
	M. packardii	2.79	(84)	3.95	(128)
	M. infantilis	2.83	(85)	0.74	(24)
	M. gladstoni	0.07	(2)	0.40	(13)
	M. femurrubrum	3.22	(97)	6.35	(206)
	M. confusus	0.43	(13)	0.40	(13)
	M. lakinus	0		0.10	(3)
	M. keeleri	0		0.03	(1)
	M. dawsoni	0.23	(7)	0	
	Phoetaliotes nebrascensis	10.57	(318)	0.43	(14)
Total for Melanoplinae		53.89	(1621)	40.19	(1303)
Oedipodinae	*Camnula pellucida*	35.80	(1077)	14.31	(464)
	Spharagemon collare	0.03	(1)	0.03	(1)
	S. equale	0.10	(3)	0.03	(1)
	Metator pardalinus	0		0.06	(2)
	Trachyrhachys kiowa	0.07	(2)	0.06	(2)
	Xanthippus corallipes	0		0.19	(6)
	Arphia conspersa	0		0.06	(2)
Total for Oedipodinae		36.00	(1083)	14.74	(478)
Gomphocerinae	*Ageneotettix deorum*	4.32	(130)	28.23	(915)
	Aulocara ellioti	0.23	(7)	15.52	(503)
	A. femoratum	0		0.12	(4)
	Aeropedellus clavatus	2.56	(77)	1.14	(37)
	Chorthippus curtipennis	2.99	(90)	0.06	(2)
Total for Gomphocerinae		10.11	(304)	45.07	(1461)
Total all grasshoppers		100.00	(3008)	100.00	(3242)

Table 2 Infection by pathotypes 1, 2 or 3 in grasshopper species at Field 1 and Wold's site in 1992. First value is the number of grasshoppers that were infected and the number in parantheses is the percentage of individuals collected and caged within that species that were infected

Subfamily Species	Field 1 Pathotype 1	Field 1 Pathotype 2	Field 1 Pathotype 3	Wold's Pathotype 1	Wold's Pathotype 2	Wold's Pathotype 3
Melanoplinae						
Melanoplus sanguinipes	2 (0.22)	10 (1.14)	3 (0.34)	0	43 (7.43)	7 (1.2)
M. bivittatus	4 (2.96)	2 (1.48)	0	2 (0.62)	64 (19.88)	11 (3.42)
M. packardii	0	5 (5.95)	3 (3.57)	0	16 (12.50)	6 (4.69)
M. infantilis	1 (1.18)	2 (2.35)	0	0	2 (8.33)	2 (8.33)
M. femurrubrum	1 (1.03)	1 (1.03)	3 (3.09)	0	2 (0.97)	3 (1.46)
M. confusus	0	2 (15.38)	0	0	0	0
Oedipodinae						
Camnula pellucida	33 (7.11)	6 (1.29)	10 (2.16)	128 (11.88)	5 (0.46)	31 (2.88)
Gomphocerinae						
Ageneotettix deorum	6 (4.62)	1 (0.77)	2 (1.54)	4 (0.44)	1 (0.11)	7 (0.77)
Aulocara ellioti	1 (14.29)	0	0	3 (0.60)	9 (1.80)	4 (0.80)
Aeropedellus clavatus	2 (2.59)	0		0	1 (2.70)	0 0

exhibited higher levels of pathotype 1 infections than males, but no other sexual differences in pathotype infections were noted. The early instar *M. bivittatus* exhibited higher infection frequencies with pathotype 2 at the Wold's site (Fig. 1). At Field 1 pathotype 2 infections were generally lower than at the Wold's site.

C. pellucida infections with *E. grylli* showed a trend different to that of *M. sanguinipes*. In *C. pellucida*, infections were predominant in the late instar and adult grasshoppers (Fig. 1).

In summary, four general trends are shown with respect to grasshopper life stage and subfamily infection patterns: (i) pathotype 1 predominantly infected oedipodine and gomphocerine grasshoppers and pathotype 2 predominantly infected melanopline grasshoppers, (ii) early instar *M. sanguinipes* and *M. bivittatus* had highest pathotype 2 infection frequencies, while late-instar and adult *C. pellucida* had highest pathotype 1 infection frequencies, (iii) pathotype 3 infections occurred in later instar and adult grasshoppers (iv) pathotype cross-infection (e.g. pathotype 1 infection in melanopline grasshoppers) occurred in later instar and adult grasshoppers. There is infection predominance based on host subfamily by the *E. grylli* pathotypes but cross-infection does occur in up to 3% of the infected grasshoppers within a species.

Discussion

The zygomycetous fungus *E. grylli* is composed of at least three pathotypes of grasshopper pathogens. The pathotypes show infection predominance based on host grasshopper subfamily (Ramoska *et al*. 1988) and can be discriminated by isozyme analysis (Soper *et al*. 1983), restriction fragment length polymorphisms (RFLP; Walsh *et al*. 1990), random amplification of polymorphic DNA (RAPD) by the polymerase chain reaction (PCR) and pathotype specific DNA probes (Bidochka *et al*. 1995). Most recently, DNA probes had been used to track the fate of the Australian pathotype that was released in North Dakota (Bidochka *et al*. 1996). DNA probes were applied in discriminating pathotypes where otherwise they could not be identified using conventional methods such as morphological traits.

In 1992 pathotype 3 was found in ≈ 23% of all *E. grylli*-infected grasshoppers at the release sites but in 1993 pathotype 3 levels dropped to less than 2% of all *E. grylli*-infected grasshoppers (Bidochka *et al*. 1996). Here we have shown that *E. grylli* infections are variable with respect to the grasshopper life stages.

The application of DNA probes to differentiate the three *E. grylli* pathotypes was instrumental in assessing infections in the grasshopper life stages under field conditions. Early-instar *M. sanguinipes* and *M. bivittatus* had higher pathotype 2 infection frequencies, while late instar and adult *C. pellucida* had higher pathotype 1 infection frequencies. And pathotype 3 infections occurred in late-instar and adult grasshoppers. It is difficult to resolve the reasons for the differences in the life-stage distribution of grasshopper infections by the three pathotypes because interactions between grasshopper life stages, fungal life cycles and, environmental factors are complex. With respect to fungal life cycles, all pathotypes produce resting spores in infected grasshoppers. However, only pathotypes 1 and 3 produce conidia. Pathotype 2 produces cryptoconidia and horizontal disease transmission by these spores is considered to be minimal (Humber & Ramoska 1986). Because pathotype 2 does not produce conidia, its infection cycle is monocyclic, limited to a single season and is relatively short-lived in comparison to pathotype 1. The production of conidia by pathotypes 1 and 3 stimulates secondary infection cycles (Carruthers & Soper 1987). The association of pathotypes 1 and 3 with infections in adult or later-instar grasshoppers could be due to the conidia produced in enzootic infections which subsequently infected grasshoppers later in the season to produce an epizootic; presumably later in the grasshopper life cycle. The association of pathotype 2 primarily with early-instar *M. sanguinipes and M. bivittatus* supports the observation that infections by pathotype 2 are monocyclic.

Laboratory investigations have previously shown that pathotype 1 predominantly infected oedipodine grasshoppers and pathotype 2 infected melanopline grasshoppers (Ramoska *et al*. 1988). The present study confirms these findings with results from the field. We have also shown that pathotype 1 predominantly infected gomphocerine grasshoppers. However, up to 3% of grasshoppers within a species showed pathotype cross-infection. Laboratory bioassays have shown that cross-infection of pathotype 1 in melanopline grasshoppers does occur, but rarely; however, pathotype 2 infections were not found in oedipodine grasshoppers (Ramoska *et al*. 1988). In our study, when cross-infections were detected in nature, the frequency of association was highest for pathotype 1 infections in melanopline grasshoppers. In this case the laboratory and field results coincide. However, in laboratory tests, it was the grasshopper cuticles that were exposed to conidia of pathotype 2 or, the grasshoppers were injected intrahemocoelically with protoplasts (pathotype 1) or conidia (pathotype 2) (Ramoska *et al*. 1988). In the natural environment, other routes of infection are possible. For example, grasshoppers are frequently necrophagous (Lockwood 1988, 1989) and may consume conidia or resting spores associated with dead, *E. grylli*-infected, grasshoppers. Grasshoppers may become wounded during moulting or otherwise and this also provides an opportunity for infection. These alternate routes of infection may be responsible for the cross-infections observed in the field and may

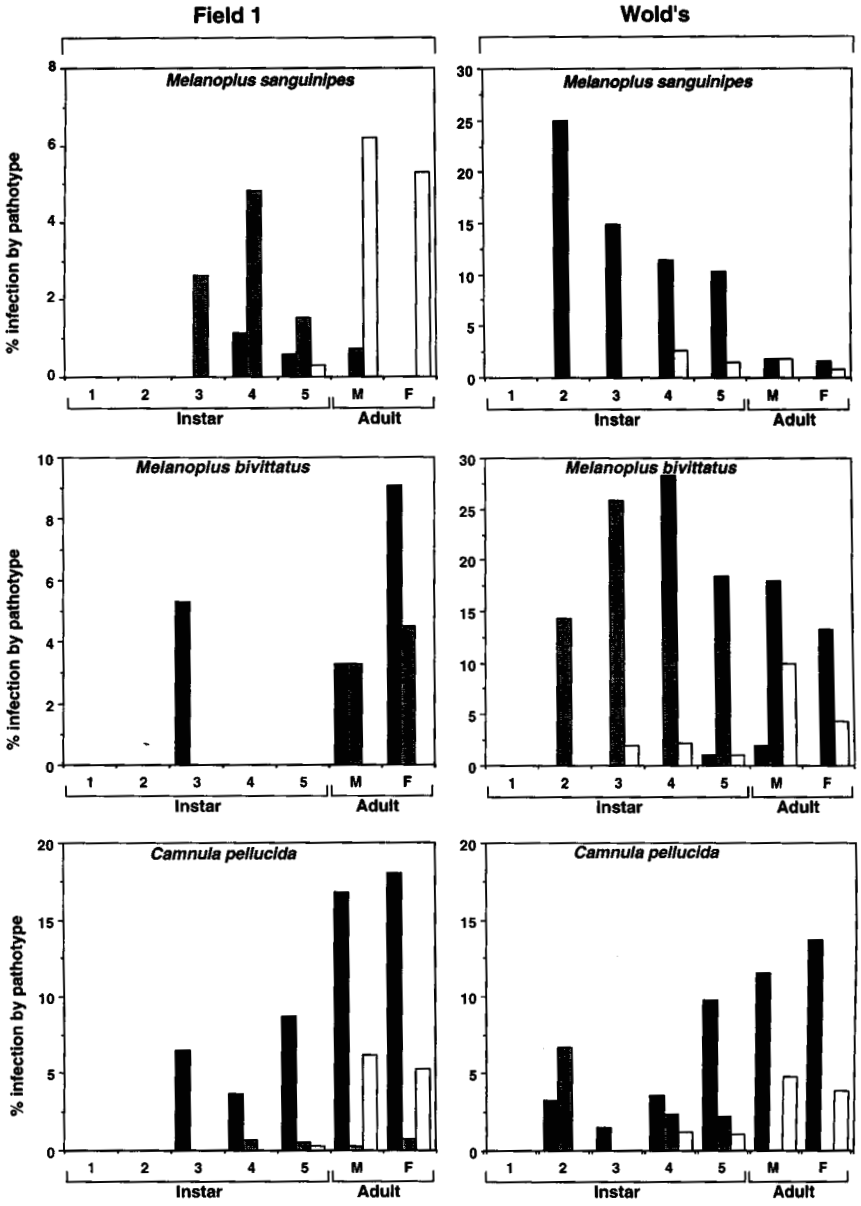

Fig. 1 Distribution of *Entomophaga grylli* pathotype infections in life stages of *Melanoplus sanguinipes*, *M. bivittatus* and *Camnula pellucida* collected at the Field 1 and Wold's sites; instars 1–5 and adult females and males; pathotype 1 (■), pathotype 2 (▒) and pathotype 3 (□).

account for the observation of pathotype 2 infections in oedipodine grasshopper that were not observed in the laboratory. However, to our knowledge, there are no data supporting oral or other routes of fungal infection, other than transcuticular, of grasshoppers in the field.

Pathotype cross-infections appeared in later-instar and adult grasshoppers, and when cross-infections occurred they were generally found in melanopline grasshoppers infected by pathotype 1. Cross-infection by pathotype 1 is coincident with the ability of pathotype 1 to produce secondary infections through the production of conidia. The conidia produced in already infected oedipodine grasshoppers could infect melanopline grasshoppers. However, the mechanisms by which host-specificity operates in *E. grylli* still remain to be elucidated. Most mechanisms limiting *E. grylli* infection in non-host grasshoppers are presumed to be those effective prior to invasion of the haemocoel and include cuticular cues for conidial or resting spore adhesion, germination, and penetration. Another pre-invasion factor that may play a role is host phenology. Post-invasion factors that may limit infection include host immune responses and behavioural actions, specifically sun basking known as 'behavioural fever' (Carruthers *et al*. 1992). What leads to the breakdown of the factors that limit cross-infection is not known.

Clearly, the utilization of molecular methods for evaluating ecological interactions is becoming increasingly necessary. This is particularly true in the study of certain obligate pathogens such as the *E. grylli* pathotypes where morphological features are indiscriminate. In this study

infection preferences by the *E. grylli* pathotypes were observed in life stages of grasshopper species. We have also shown that cross-infection in non-preferred hosts can also occur in the field. Together, this information gives a clearer understanding of host–pathogen dynamics in a prairie ecosystem particularly when the pathogen component is augmented by the introduction of an exotic fungal species.

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